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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The purpose of this study was to begin to understand the mechanism of replication initiation in mammalian cells in order to gain insight into how misregulation of initiation may lead to cancer progression. We have shown that a 5.8 kb DNA fragment containing the initiation region (IR) DHFR ori-β is active at ectopic chromosomal locations in hamster cells and that deletion of three specific elements in ori-β reduced initiation activity. Further characterization of these elements showed that an AT-rich element is required for efficient ori-β activity, and that a homologous region of the human lamin B2 origin can substitute for this element. In addition, the 5.8 kb ori-β DNA fragment is sufficient to direct replication initiation in two human cancer cell lines, suggesting a possible conservation of the mechanisms of replication initiation in mammalian cells. In order to begin to understand the relationship of origin activity and initiator protein binding, we assessed protein binding to ectopic ori-β in a human cancer cell line. We found that initiation proteins localized to the replication start site of ori-β and deletion of the AT-rich element modulated protein binding. Taken together, these results suggest a potential relationship between ORC binding and origin selection and activation.

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Abstract

Mapping of replication start sites in several loci in mammalian chromosomes has revealed that initiation begins at a few high frequency start sites contained in a broad zone of initiation. We have shown that a 5.8 kb DNA fragment containing the high frequency initiation region (IR) DHFR ori-β is active at multiple ectopic chromosomal locations in hamster cells and that deletion of three specific elements in ori-β reduced initiation activity (Altman and Fanning, 2001). Deletion of an AT-rich element reduced initiation activity to background levels. However, an AT-rich region from the human lamin B2 IR was able to substitute for the DHFR ori-β AT-rich sequence and actually enhanced ori-β initiation activity over 10-fold. Replacement of the required AT-rich element with a similar sized non-origin DNA fragment was not able to restore initiation activity. The enhancement of initiation activity was not due to the presence of a second initiation start site since the laminB2 fragment, although containing the mapped origin of replication, did not function as a start site for replication in the ori-β context. Interestingly, deletion of 7 bp within the laminB2 replacement element reduced initiation levels to about 1/3 of that of the replacement construct. Taken together, these results suggest that the AT-rich element is absolutely required for efficient ori-β activity.

The ability of a human DNA element to function in a hamster origin of replication initiation raises the question of whether a hamster origin may function in human cells. Recent work shows that the 5.8 kb ori-β DNA fragment is sufficient to direct replication initiation in human (Hela and HCT116) cells, suggesting a possible conservation of the mechanisms of replication initiation in mammalian cells. In addition, the results of the AT-rich element analysis were reproducible when the mutants were tested in the Hela cell background. In an effort to begin to understand the relationship of origin activity and initiator protein binding, a chromatin immunoprecipitation (ChIP) assay was used to assess protein binding to the ori-β IR located in ectopic chromosomal locations in G1 phase Hela cells. We found that members of the pre-replicative complex (preRC) localized to the mapped replication start site of ori-β and deletion of the AT-rich element modulated pre-RC binding. Taken together, these results suggest a potential relationship between ORC binding and origin selection and activation.

Research Summary

INTRODUCTION

Review of proposal relevance

The initiation of DNA replication in eukaryotic chromosomes represents one of the crucial events in the control of the cell division cycle. It is believed that this control is exerted primarily at the initiation of DNA replication at the thousands of origins spread across the genome. Each of the roughly 50,000 to 100,000 origins must be tightly regulated to maintain the integrity of the genome and to ensure that replication at each origin occurs only once per cell cycle. During normal development in some organisms, multiple rounds of replication at the same origin lead to an amplification of specific genomic sequences (Carminati et al. 1996, Breur et al. 1955, Liang et al. 1993, Spradling et al. 1999 and referenced therein). Under certain conditions, such as mutations in the p53 tumor suppressor gene, amplification can also occur in the human genome, and has been implicated as a prognostic factor in a number of cancers, including breast cancer (Slamon et al. 1987, Wright et al. 1989, Bonilla et al. 1988, Zhou et al. 1988, McWhinney and Leffak 1990, Vassilev and Johnson 1990, Tlsty et al. 1989, Tlsty 1990, Livingstone et al. 1992, Yin et al. 1992). Given that some amplification can be the result of overcoming the block to rereplication at origins of replication, it is imperative to understand the control of replication initiation in mammalian chromosomes in order to gain a better understanding of the role of amplification in breast cancer development and progression. Once the cis-acting sequences involved in DNA replication initiation are understood, their interaction with proteins that regulate replication initiation and amplification can be investigated.

One of the major gaps in our understanding of mammalian DNA replication is a genetic definition of a chromosomal origin of DNA replication. Initiation of DNA replication in *Escherichia coli*, mammalian viruses, and the budding yeast *Saccharomyces cerevisiae* (DePamphilis 1996, Newlon 1996) is controlled primarily by trans-acting initiator proteins that interact with a cis-acting DNA sequence element (the replicator) (Jacob et al. 1963). Since many of the initiator proteins found in yeast appear to be conserved in higher eukaryotes (reviewed in Dutta and Bell. 1997), it is possible that DNA replication initiation in mammalian cells may also be directed by a replicator and occur site-specifically within an initiation region (IR). Extensive mapping of replication start sites in mammalian chromosomes has revealed that replication at most, but not all, loci begins at a few high frequency start sites contained within a broad zone of initiation (reviewed in Gilbert 1998, DePamphilis 1999, Spradling 1999, Bogan et al. 2000, Toledo et al. 1998). One of the most thoroughly mapped high frequency initiation regions (IRs) in mammalian chromosomes is the region downstream from the dihydrofolate reductase (DHFR) gene in Chinese Hamster Ovary (CHO) cells (Fig. 1A). This region contains a 55

kb zone of delocalized origin activity containing three preferred start sites: ori-β centered approximately 17 kb downstream from the DHFR gene, ori-β' just downstream from ori-β, and ori-γ located 23 kb further downstream (Heintz and Hamlin 1982, Heintz et al. 1983, Anachkova and Hamlin 1989, Handeli et al. 1989, Leu and Hamlin 1989, Burhans et al. 1990, Vassilev et al. 1990, Vaughn et al. 1990, Pelizon et al. 1996, Kobayashi et al 1998, Wang et al. 1998 Li et al. 2000).

Despite the progress in mapping initiation sites, the specific genetic elements necessary to direct initiation of mammalian DNA replication remain poorly understood. The existence of preferred IRs raises the question of whether specific DNA sequences within or neighboring an IR may direct initiation of replication at the IR or even in a broad zone more distant from the preferred IR. To address the question of whether initiation of DNA replication can be directed by specific sequences flanking the single preferred IR ori-β of the DHFR locus, we have stably transfected an exogenous DNA fragment containing the ori-β IR in random ectopic chromosomal locations and assayed for initiation activity by using a competitive polymerase chain reaction (PCR)-based nascent strand abundance assay.

RESEARCH SUMMARY

The main goal of the research proposal was to develop an assay system for studying the specific genetic elements, if any, involved in the initiation of DNA replication in mammalian cells as outlined in Task 1 (development of assay system, see Appendix Statement of Work). The completion of this task is outlined in the annual summary from the first year and led to the first peer-reviewed publication resulting from this research proposal (See Annual Summary, 2000, and attached reprint Altman and Fanning, 2001). Briefly, a competitive polymerase chain reaction (PCR)-based nascent strand abundance assay was used to demonstrate the ability of a small 5.8 kb fragment of DNA, containing the DHFR ori-β initiation region (IR), to support efficient origin activity when integrated into random ectopic positions in the hamster chromosome (Altman and Fanning, 2001). In addition, the integrated ori-β IR functioned with the same efficiency as the endogenous ori-β in CHOK1 cells, suggesting that the 5.8 kb fragment was an acceptable candidate for mutational analysis of the ori-β IR.

Studies of model systems such as animal viruses (SV40, Epstein-Barr virus, herpes simplex virus), mitochondria (human, mouse), protozoa (*Tetrahymena*) and yeast (*S. cerevisiae, Schizosaccharomyces pombe*), have determined that origins have a modular organization composed of unique DNA sequence motifs and interactions with soluble proteins (reviewed in DePamphilis, 1993). Task 4 (identification of essential cis-acting sequences) of the research proposal was designed to test whether complex mammalian origins also have a modular organization of specific cis-acting DNA sequence elements which are essential for the initiation of DNA replication. The attached reprint (Altman and

Fanning, 2001) successfully addressed Task 4 and details the deletion of four specific DNA sequence elements within the 5.8 kb DNA fragment which show sequence similarity to modular elements found in characterized origins. Briefly, deletion of three of these putative elements led to a significant decrease in initiation activity, as assayed by the PCR-based nascent strand abundance assay, whereas a fourth element appeared to be dispensable for initiation activity. These results suggested that, indeed, there are specific genetic elements that are necessary for efficient initiation of DNA replication in a mammalian origin. However, it is possible that these deletions affected initiation by changing the spacing of important flanking elements.

In order to confirm that the sequences deleted above were true cis-acting sequence modules required for initiation, a series of substitution mutations were created that addressed this issue. One of the four sequences deleted in the described mutation analysis, the downstream AT-rich region (Altman and Fanning, 2001, Fig. 4A) showed some sequence homology with a cell cycle-dependent protein binding site in the human lamin B2 IR (Giacca et al, 1994; Dimitrova et al., 1996; Abdurashidova et al., 1998). To determine whether this region of the lamin B2 IR was able to substitute for the DHFR ori-β AT-rich domain, several mutant constructs were created and analyzed using the PCR-based nascent strand abundance assay. Initiation activity of the lamin B2 replacement construct was actually increased compared to the wild type 5.8 kb DHFR ori-β fragment, whereas replacement of the AT-rich domain with a similar sized non-origin DNA fragment from the neomycin resistance gene showed initiation levels similar to the AT-rich deletion construct, suggesting that the decrease in initiation seen in the AT-rich deletion construct was not due to a change in fragment size or spacing of flanking DNA sequence elements. In addition, deletion of 7 bp within the lamin B2 replacement construct led to a decrease in activity compared to the lamin B2 replacement alone. These results suggest that the AT-rich domain represents an essential DNA sequence element and that this sequence element may be conserved in mammalian origins. A small 4 bp deletion within the DHFR ori-β region also led to a decrease in activity (Altman and Fanning, 2001). To determine whether this decrease in initiation activity was due to a specific sequence or due to spacing issues, the 4 bp 5'-GGCC sequence was replaced with 5'-CATG. This 4 bp substitution construct was unable to restore initiation activity to the 4 bp deletion construct, suggesting that the 4 bp sequence is important for initiation, perhaps by being a part of a protein binding motif. The identification of essential DNA sequence elements in the DHFR ori-β IR provides support for a modular organization of mammalian origins. These results expand on Task 4 (identification of cis-acting sequences) in the statement of work and provide important insights into the organization of mammalian origins. This work is will be included in the second peer-reviewed publication from this work (Altman and Fanning, *in preparation*). Further characterization of the DHFR ori-β IR though mutational analysis is worthwhile to identify and confirm additional essential DNA sequence elements in order to better understand the mechanism of origin selection and activation in mammalian chromosomes.

As outlined in the review of proposal relevance, many cancers, including breast cancer, may arise from faulty regulation of the DNA replication initiation process at certain origins which leads to amplification of genomic regions. In order to gain a better understanding of the role of amplification in breast cancer development and progression, it was important to assess replication initiation in a few cancer cell lines. Therefore, Task 3 in the statement of work (assay ori-β in human cancer cell lines) was to establish the PCR-based nascent strand abundance assay in human tumor cell lines. Briefly, the 5.8 kb DNA fragment, containing the DHFR ori-β IR, was transfected into HeLa cells and HCT116 cells. HeLa cells are a cervical cancer cell line and HCT116 cells are a colon cancer cell line. Transfection of the exogenous DNA into the human tumor cell lines was optimized to achieve integration of approximately 2 copies of the DHFR ori-β fragment per cell. In addition, it was confirmed that the established primer sets were specific for the DHFR ori-β IR and showed no amplification from untransfected HeLa or HCT116 genomic DNA. Initiation activity of the DHFR ori-β IR in random ectopic locations in the human chromosome was assayed with the PCR-nascent strand abundance assay in pools of transfected cells. The 5.8 kb fragment was sufficient to direct efficient initiation from ori-β in both of the human tumor cell lines. In fact, replication initiation activity was actually increased about 2 fold compared to initiation at ori-β in the DR12 hamster cell line. It is possible that the increased initiation activity of ori-β seen in the tumor cell lines is due to specific differences inherent to tumor cell lines and may provide insight into the loss of cell cycle control which is the hallmark of cancer progression. This result is important in that it demonstrates that a hamster origin is able to function efficiently in a human cell line, suggesting a possible conservation of origin structure among mammalian cells. In addition, these results suggest that all of the necessary soluble protein factors needed to identify and activate ori-β are present in the human cell line implying possible conservation of the mechanisms of replication initiation. This idea is supported by the fact that the ori-β deletion constructs behaved similarly in the human cancer cell lines as they did in the hamster background. The ability of the 5.8 DHFR ori-β fragment to support efficient initiation in human cancer cell lines, and the fact that deletions within the fragment have deleterious effects on initiation, provides a perfect model system to test the interaction of initiator proteins with origins of replication. In fact, preliminary studies in our lab show that deletion of the essential AT-rich element modulates binding of the initiation protein complex at ectopic ori-β in Hela cells (Patten, Altman and Fanning, unpublished data). These studies address Task 3 (assay ori-β in human cancer cell lines) and provide an important model system for studying the mechanisms of replication initiation in a human tumor cell line.

In summary, the studies outlined above successfully address Task 1 (development of assay system), Task 4 (identification of essential cis-acting sequences), and Task 3 (assay ori-β in human cancer cell lines). The identification and characterization of specific cis-acting sequence elements essential for replication initiation (task 4) represents one of

the most important aspects of this research proposal in that it provides, for the first time, an idea of what constitutes a mammalian origin. Therefore, significant effort has been made to confirm the existence of modular sequence elements within the DHFR ori-β IR. Task 2 in the statement of work was to address the affects of linking the neomycin gene to the ectopic ori-β. However, studies by other groups have found that transcription activity near an origin can have a significant affect on origin selection and activation. For example, a deletion that abolished transcription at the DHFR gene abrogates replication at the ori-β IR (Hamlin and Dijkwel, 1995). In addition, work on the beta-globin IR has demonstrated that replication timing depends of the transcriptional status of the integration locus (Aladjem et al., 2002). In order to assess the true role that specific sequences have in origin selection, it was important to test initiation activity at ori-β free from other contributing factors, such as local transcriptional activity, and therefore task 2 was not initiated. Task 5 in the statement of work (testing activity of ori-β mutations in endogenous locus) was also not initiated due to the fact that this very experiment is being conducted in another laboratory (Kolman and Wahl, personal communication). Rather than being redundant, I have chosen to focus on the characterization of modular sequence elements (Task 1, 3, and 4). It is important to note that this work represents the first evidence of the importance of specific cis-acting sequence elements in mammalian replicators. Therefore, I continued to characterize modular sequence elements and our lab has begun to study their interaction with initiator proteins. Given the success of the established assay system in both human and hamster cell lines, and the potential for these studies to bear fruit, Tasks 1, 3 and 4 of the original statement of work have taken precedence over Task 2 and 5.

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APPENDIX

Approved Statement of Work from Grant Proposal

Task 1. To develop and further characterize an assay to identify DHFR DNA sequences able to direct initiation of chromosomal DNA replication (Months 1-8)

- Transfect DR12 cell line with 5.8 kb DHFR origin region fragment and isolate cell clones
- Confirm exogenous DHFR ori-β incorporation by PCR analysis
- Perform competitive PCR to establish DHFR ori-β function in the DR12 cell clones
- Conclude whether the 5.8 kb fragment of the DHFR ori-β region is sufficient to support origin function *in vivo* in multiple chromosomal sites

Task 2. To test whether linking the neomycin resistance gene to the 5.8 kb DNA fragment alters the initiation activity (Months 4-10)

Task 3. To establish the PCR-based assay in normal human and tumor cell lines by transfecting the 5.8 kb DHFR origin fragment into Hela cells, IMR90 human fibroblast cells, and MCF7 human breast cancer cells and assaying for origin function (Months 6-14)

Task 4. To test whether specific *cis*-acting sequences are essential for the initiation of DNA replication at the DHFR ori-β region (Months 6- 36)

- Create additional large deletion mutations within the 5.8 kb DHFR ori-β region
- Electroporate DHFR ori-β deletion mutants into the DR12 cell line
- Confirm exogenous DHFR ori-β incorporation through PCR analysis of transfected DR12 cell pools
- Perform competitive PCR to characterize origin function of DHFR ori-β mutants
- If certain regions appear to be essential for initiation activity, fine map sequences within regions of interest through point mutation and analysis

Task 5. Insert exogenous mutations into endogenous locus in place of endogenous origin and test for origin function (Months 12-36)

Key Research Accomplishments

Annual Summary 2000

- Development of a PCR-based nascent strand abundance assay for determining the initiation activity of exogenous DNA fragments containing the DHFR ori-β IR.
- Development of normalization methods to permit comparison between independent transfection experiments.
- Demonstration that a 5.7 kb fragment containing the DHFR ori-β IR is sufficient to direct initiation of DNA replication in the locus.
- Identification of three specific cis-acting sequence elements required for efficient initiation of DNA replication from DHFR ori-β.
- Identification of one specific cis-acting sequence element that is dispensable for efficient initiation of DNA replication from DHFR ori-β.

Annual Summary 2001

- Determination that a DNA sequence element from the human lamin B2 IR is able to substitute for an AT-rich region of the DHFR ori-β IR.
- Determination that a non-origin DNA sequence element from the neomycin resistance gene is unable to substitute for an AT-rich region of the DHFR ori-β IR.
- Establishment of the PCR-based nascent strand abundance assay in a human tumor cell line.
- Demonstration that a 5.8 kb fragment containing the DHFR ori-β IR is sufficient to direct initiation of DNA replication at random ectopic locations in HeLa human tumor cells.

Annual Summary 2002

- Determination that specific sequences, such as the AT-rich region and the 4 bp within the mapped IR, are required for efficient initiation at ectopic ori-β.

- Determination that a homologous sequence element from the lamin B2 is able to substitute for the AT-rich ori-β element, and deletion of 7 bp within this fragment decrease initiation activity, suggesting that modular sequence elements are required and are conserved among mammalian origins.

- Demonstration that a 5.8 kb fragment containing the DHFR ori-β IR is sufficient to direct initiation of DNA replication at random ectopic locations in both HeLa and HCT116 human tumor cell lines.

- Demonstration that deletion mutations of the ori-β IR fragment behave similarly in human cancer cell lines as in the hamster cell milieu.

- Demonstration that the AT-rich sequence element of ori-β modulates initiator protein binding to the origin.

Reportable Outcomes

(for entire performance period)

Abstracts

The Salk Institute Eukaryotic DNA Replication meeting, The Salk Institute of Biological Sciences, La Jolla, CA. August, 2002.
POSTER: "A functional element conserved among mammalian chromosomal origins of replication."

Era of Hope Department of Defense Breast Cancer Research Program Meeting, Orange County Convention Center, Orlando, FL. September 2002.
POSTER: "Genetic Definition of a mammalian origin of replication"

Cold Spring Harbor Eukaryotic DNA Replication Meeting, Cold Spring Harbor, NY. September, 2001.
ORAL PRESENTATION: "An element from the human lamin B2 origin restores initiation activity to a DHFR ori-β deletion mutant."

The Salk Institute Eukaryotic DNA Replication meeting, The Salk Institute, La Jolla, CA. September, 2000.
ORAL PRESENTATION: "The Chinese hamster DHFR replication origin beta is active at multiple ectopic chromosomal locations and requires specific DNA sequence elements for activity."

Cold Spring Harbor Eukaryotic DNA Replication meeting, Cold Spring Harbor, NY. September, 1999.
POSTER: "Specific sequences are required to direct initiation of DNA replication in mammalian chromosomes."

Penn State's 18th Summer Symposium in Molecular Biology: Chromatin Structure and DNA Function. Penn State, State College, PA. July 1999.
POSTER: "Specific DNA sequences are required to direct initiation of DNA replication in mammalian chromosomes."

Publications

Altman, A. L. and Fanning, E. 2001. The Chinese hamster dihydrofolate reductase replication origin beta is active at multiple ectopic chromosomal locations and requires specific DNA sequence elements for activity. Mol. Cell. Biol. **21:** 1098-1110.

Altman, A. L., and Fanning, E. 2002. A functional element conserved among mammalian chromosomal origins of replication. *In preparation.*

Patten, A., Altman, A. L.*, and Fanning, E. 2002. Deletion of an AT-rich element from ectopic DHFR ori-β causes mislocalization of ORC binding and loss of origin function. *In preparation.* (*co-first author)

Career Development

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A FUNCTIONAL ELEMENT CONSERVED AMONG MAMMALIAN CHROMOSOMAL ORIGINS OF REPLICATION

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Mapping of replication start sites in several loci in mammalian chromosomes has revealed that initiation begins at a few high frequency start sites contained in a broad zone of initiation. We have shown that a 5.8 kb DNA fragment containing ori-β, a high frequency initiation region (IR) from the hamster DHFR locus, is active at multiple ectopic chromosomal locations in hamster cells (Altman and Fanning, Mol. Cell. Biol. **21:**1098, 2001). Three elements identified by deletion analysis, including an AT-rich element, were required for full initiation activity of ectopic ori-β. An AT-rich region from the human lamin B2 IR substituted for the ori-β AT-rich element and actually enhanced ori-β initiation activity by over 10-fold. A lamin B2 AT-rich element with a 7 bp deletion also substituted for the ori-β AT-rich element, but did not greatly enhance ori-β activity. Replacement of the ori-β AT-rich element with a similar sized non-origin DNA fragment did not support initiation activity of ori-β. In the context of ori-β, start sites in the lamin B2 AT-rich element were not observed, even though this element contains the mapped lamin B2 IR. Taken together, the results suggest that specific sequences in the AT-rich element are required for efficient ori-β activity and that elements from different mammalian origins may be functionally conserved. The sequence-specific requirement for the other ori-β elements is currently under investigation.

The ability of a human DNA element to function in a hamster origin of replication suggests that a hamster origin might function in human cells. Indeed, the ectopic 5.8 kb ori-β DNA fragment was sufficient to direct initiation in human (Hela and HCT116) cells. Moreover, mutational analysis confirmed that the sequences in the AT-rich element were also required for ori-β activity in the human cell background. To explore the relationship between ori-β activity and initiator protein binding, proteins bound to ori-β at ectopic chromosomal locations in G1 phase Hela cells were assayed by chromatin immunoprecipitation (see Patten abstract). We found that proteins of the pre-replicative complex (pre-RC) were localized near the mapped replication start site of ori-β and that deletion of the AT-rich element caused mislocalization of pre-RC proteins. Taken together, these results suggest that the AT-rich element plays a role in directing pre-RC proteins to a potential start site. (Supported by NIH, the Army Breast Cancer Program, UNCF-Merck, and Vanderbilt University).

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GENETIC DEFINITION OF A MAMMALIAN ORIGIN OF REPLICATION

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The initiation of DNA replication in the eukaryotic chromosome represents one of the crucial events in the control of the cell division cycle. Each of the roughly 50,000 to 100,000 origins must be tightly regulated to maintain the integrity of the genome and to ensure that replication at each origin occurs only once per cell cycle. During normal development in some organisms, multiple rounds of replication at the same origin lead to an amplification of specific genomic sequences. Under certain conditions, such as mutations in the p53 tumor suppressor gene, amplification can also occur in the human genome, and has been implicated as a prognostic factor in a number of cancers, including breast cancer. Given that amplification seems to be the result of overcoming the block to rereplication at origins, it is imperative to understand the control of replication initiation in mammalian chromosomes and how it pertains to breast cancer development and progression.

To begin to identify the elements essential for DNA replication initiation in mammalian chromosomes, we have stably transfected a 5.8 kb fragment of DNA containing the dihydrofolate reductase (DHFR) ori-β initiation region (IR) into random ectopic chromosomal locations in a hamster cell line lacking the endogenous DHFR locus. Using a competitive PCR-based nascent strand abundance assay, initiation at ectopic ori-β in pools of transfected cells and in cloned cell lines was shown to mimic, qualitatively and quantitatively, initiation at the endogenous ori-β in CHOK1 hamster cells. To assess the importance of specific DNA sequences for initiation activity, a panel of ori-β deletion mutants was constructed and assayed in the same manner. Deletion of an extensive GA dinucleotide repeat or an AT-rich region reduced initiation activity to near background levels. Deletion of 4 bp between two AT-rich regions also led to a modest decrease in initiation activity. These results suggest that a 5.8 kb fragment of DHFR ori-β is sufficient to direct initiation at ectopic chromosomal locations and that specific elements are required for efficient initiation activity. To test whether the hamster cell milieu is essential for ori-β function, we placed the 5.8 kb fragment containing the ori-β IR at ectopic chromosomal locations in a human HeLa cancer cell line. Initiation activity of the ori-β IR was actually several-fold higher in the HeLa background than in the hamster background. To assess whether specific DNA sequence elements from human origins of replication can substitute for hamster DNA sequence elements, we replaced the required AT-rich region of the DHFR ori-β IR with an AT-rich region of the human lamin B2 IR and found it was able to restore and actually enhance initiation activity at ori-β in hamster cells. However, replacement of the AT-rich sequence with a similar sized, non-origin DNA fragment did not support initiation activity. Taken together, these results suggest that mammalian origins may be composed of modular elements that are functionally conserved among different origins. This work provides the first evidence of required cis-acting elements in mammalian origins.

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AN ELEMENT FROM THE HUMAN LAMIN B2 ORIGIN RESTORES INITIATION ACTIVITY TO A DHFR ORI-β DELETION MUTANT.

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Mapping of replication start sites in several loci in mammalian chromosomes has revealed that initiation begins at a few high frequency start sites contained within a broad zone of initiation. We have shown that a 5.8 kb hamster DNA fragment containing the high frequency initiation region (IR) DHFR ori-β is active at multiple ectopic chromosomal locations in hamster cells and that specific deletions in the ori-β fragment compromised initiation activity (1). To test whether the hamster cell milieu is essential for ori-β function, we placed the 5.8 kb fragment containing the ori-β IR at ectopic chromosomal locations in a human HeLa cell line. We found that initiation activity of the ori-β IR was actually several-fold higher in the HeLa background than in the hamster background. This result suggests that DNA fragments containing human IRs may be active when placed at ectopic chromosomal sites in hamster cells, a possibility that is under investigation.

The ability of a hamster replicator to function in human cells raises the question of whether it may contain sequence elements functionally homologous to those of human replicators. To assess whether specific DNA sequence elements from human origins of replication can substitute for hamster DNA sequence elements, we replaced a required AT-rich region of the DHFR ori-β IR with an AT-rich region of the human lamin B2 IR that contains a cell cycle-dependent protein footprint (2). The lamin B2 IR sequence was able to substitute for the DHFR ori-β AT-rich sequence to restore and actually enhance its initiation activity in hamster cells. However, replacement of the AT-rich sequence in the 5.8 kb DHFR ori-β fragment with a similar sized, non-origin DNA fragment did not support initiation activity. Taken together, these results suggest that mammalian origins may be composed of modular elements that are functionally conserved among different origins. (Supported by NIH GM 52948, the Army Breast Cancer Program BC980907, and Vanderbilt University).

1) Altman, A. L. and Fanning, E. (2001). The Chinese hamster dihydrofolate reductase replication origin beta is active at multiple ectopic chromosomal locations and requires specific DNA sequence elements for activity. Mol. Cell. Biol. **21:** 1098-1110.

2) Dimitrova, D. S., Giacca, M., Demarchi, F., Biamonti, G., Riva, S., and Falaschi, A. (1996) In vivo protein-DNA interactions at a human DNA replication origin. Proc. Natl. Acad. Sci. USA **93:** 1498-1503.

The Salk Institute Eukaryotic DNA Replication meeting, The Salk Institute
La Jolla, September, 2000.

THE CHINESE HAMSTER DHFR REPLICATION ORIGIN BETA IS ACTIVE AT MULTIPLE ECTOPIC CHROMOSOMAL LOCATIONS AND REQUIRES SPECIFIC DNA SEQUENCE ELEMENTS FOR ACTIVITY. Amy L. Altman and Ellen Fanning, Department of Biological Sciences and the Vanderbilt-Ingram Cancer Center, Vanderbilt University, Nashville, TN 37232

Mapping of replication start sites in mammalian chromosomes has revealed that initiation at most loci begins at a few high frequency start sites contained within a broad zone of initiation. One of the most thoroughly mapped high frequency initiation regions (IRs) in mammalian chromosomes is the region downstream from the dihydrofolate reductase (DHFR) gene in Chinese Hamster Ovary (CHO) cells. This region contains a 55 kb zone of less frequent start sites and three preferred start sites. Ori-β is centered approximately 17 kb downstream from the DHFR gene, ori-β' is just downstream from ori-β, and ori-γ is located 23 kb further downstream.

Despite the progress in mapping mammalian IRs, the specific genetic elements necessary to direct initiation of mammalian DNA replication remain poorly understood. To identify the elements essential for DNA replication initiation at the DHFR ori-β preferred IR in mammalian chromosomes, we have stably transfected a 5.7 kb fragment of the DHFR locus containing the ori-β initiation region into random ectopic chromosomal locations in a hamster cell line lacking the endogenous DHFR locus. Using a competitive PCR-based nascent strand abundance assay, initiation at ectopic ori-β in pools of transfected cells and in six cloned cell lines was shown to mimic, qualitatively and quantitatively, initiation at the endogenous ori-β in CHOK1 hamster cells. To assess the importance of specific DNA sequences for initiation activity, a panel of ori-β deletion mutants was constructed and assayed in the same manner. A striking decrease in initiation activity, more than 10-fold, resulted when an extensive GA dinucleotide repeat was deleted from the distal end of the fragment. Deletion of an AT-rich region also reduced initiation activity to near background levels. Deletion of 4 bp between two AT-rich regions also led to a modest decrease in initiation activity. These results suggest (1) that a 5.7 kb fragment of DHFR ori-β is sufficient to direct initiation at ectopic chromosomal locations, (2) that within this fragment, at least two widely spaced elements of very different sequence composition are required for efficient initiation activity, and (3) that the DHFR initiation zone may be composed of multiple independent replicators. (Supported by NIH GM 52948 and CA 09385, the Army Breast Cancer Program BC980907, and Vanderbilt University.)

Cold Spring Harbor Eukaryotic DNA Replication meeting, Cold Spring Harbor, NY. September, 1999.

SPECIFIC DNA SEQUENCES ARE REQUIRED TO DIRECT INITIATION OF DNA REPLICATION IN MAMMALIAN CHROMOSOMES

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The initiation of DNA replication represents one of the crucial control points in the eukaryotic cell division cycle. Although much progress has been made in the biochemical identification of preferred replication start sites in mammalian chromosomes, the specific genetic elements required for the initiation of DNA replication remain unclear. To begin to elucidate the genetic elements essential for DNA replication initiation in vivo, a 5.7 kb fragment of the hamster DHFR ori-β preferred initiation region was stably transfected into random ectopic chromosomal locations in a CHO cell line which lacked any endogenous ori-β DNA. Using competitive PCR to measure the abundance of specific exogenous DNA sequences in nascent DNA isolated from asynchronous cell pools, we demonstrated enhanced abundance of nascent DNA centered over the preferred start site that had been mapped at the endogenous locus in CHOK1 cells. The initiation efficiency in the transfected cell pools was comparable to or greater than that of the endogenous ori-β in CHOK1 cells. Nascent DNA from cells transfected with mutant exogenous ori-β fragments demonstrated a requirement for specific DNA sequences for initiation activity. Deletion of 4 bp centered within the preferred start site reproducibly decreased initiation activity to a level similar to that in nonreplicating CHOK1 cells. Deletion of an A-T rich element, which shares sequence homology with a putative human lamin B2 origin protein binding site (Abdurashidova et al., 1998. EMBO J. 17, 2961-2969), resulted in a similar reproducible decrease in initiation activity. In contrast, large deletions of the 3′ end of the DHFR ori-β fragment caused great variation in initiation activity, suggesting that this region is required for reproducible efficient initiation of DNA replication at ori-β in ectopic sites and may represent an element which either enhances initiation activity or insulates the putative origin from local chromosomal context. Taken together, these data suggest that the 5.7 kb fragment is sufficient to direct efficient, reproducible initiation of DNA replication in vivo at DHFR ori-β in ectopic sites, and that this fragment contains several specific genetic elements which are necessary for this initiation.

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SPECIFIC DNA SEQUENCES ARE REQUIRED TO DIRECT INITIATION OF DNA REPLICATION IN MAMMALIAN CHROMOSOMES; A.L. Altman and E. Fanning, Department of Molecular Biology and the Vanderbilt-Ingram Cancer Center, Vanderbilt University, Nashville, TN 37232

The initiation of DNA replication represents one of the crucial control points in the eukaryotic cell division cycle. Although much progress has been made in the biochemical identification of preferred replication start sites in mammalian chromosomes, the specific genetic elements required for the initiation of DNA replication remain unclear. To begin to elucidate the genetic elements essential for DNA replication initiation in vivo, a 5.7 kb fragment of the hamster DHFR ori-β preferred initiation region was stably transfected into random ectopic chromosomal locations in a CHO cell line which lacked any endogenous ori-β DNA. Using competitive PCR to measure the abundance of specific exogenous DNA sequences in nascent DNA isolated from asynchronous cell pools, we demonstrated enhanced abundance of nascent DNA centered over the preferred start site that had been mapped at the endogenous locus in CHOK1 cells. The initiation efficiency in the transfected cell pools was comparable to or greater than that of the endogenous ori-β in CHOK1 cells. Nascent DNA from cells transfected with mutant exogenous ori-β fragments demonstrated a requirement for specific DNA sequences for initiation activity. Deletion of 4 bp centered within the preferred start site reproducibly decreased initiation activity to a level similar to that in nonreplicating CHOK1 cells. Deletion of an A-T rich element, which shares sequence homology with a putative human lamin B2 origin protein binding site (Abdurashidova et al., 1998. EMBO J. 17, 2961-2969), resulted in a similar reproducible decrease in initiation activity. In contrast, large deletions of the 3′ end of the DHFR ori-β fragment caused great variation in initiation activity, suggesting that this region is required for reproducible efficient initiation of DNA replication at ori-β in ectopic sites and may represent an element which either enhances initiation activity or insulates the putative origin from local chromosomal context. Taken together, these data suggest that the 5.7 kb fragment is sufficient to direct efficient, reproducible initiation of DNA replication in vivo at DHFR ori-β in ectopic sites, and that this fragment contains several specific genetic elements which are necessary for this initiation.

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The Chinese Hamster Dihydrofolate Reductase Replication Origin Beta Is Active at Multiple Ectopic Chromosomal Locations and Requires Specific DNA Sequence Elements for Activity

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To identify *cis*-acting genetic elements essential for mammalian chromosomal DNA replication, a 5.8-kb fragment from the Chinese hamster dihydrofolate reductase (DHFR) locus containing the origin beta (ori-β) initiation region was stably transfected into random ectopic chromosomal locations in a hamster cell line lacking the endogenous DHFR locus. Initiation at ectopic ori-β in uncloned pools of transfected cells was measured using a competitive PCR-based nascent strand abundance assay and shown to mimic that at the endogenous ori-β region in Chinese hamster ovary K1 cells. Initiation activity of three ectopic ori-β deletion mutants was reduced, while the activity of another deletion mutant was enhanced. The results suggest that a 5.8-kb fragment of the DHFR ori-β region is sufficient to direct initiation and that specific DNA sequences in the ori-β region are required for efficient initiation activity.

Initiation of DNA replication in *Escherichia coli*, mammalian viruses, and the budding yeast *Saccharomyces cerevisiae* (23, 67) is controlled primarily by *trans*-acting initiator proteins that interact with *cis*-acting DNA sequence elements (the replicators) (46). In these simple replicons, the *cis*-acting element consists of an essential core sequence, containing initiator protein binding sites and easily unwound sequences (DNA unwinding elements [DUEs]), and auxiliary sequences which enhance the efficiency of replication initiation (23, 67). In *S. cerevisiae*, the initiation of replication requires an autonomously replicating sequence (ARS) element in *cis* and occurs within a region flanked by a DUE and the binding sites for the origin recognition complex (ORC) and other initiator proteins (10). The initiation site for leading-strand synthesis appears to be restricted to a single nucleotide in a chromosomal ARS element (11). Replication initiation in fission yeast is also controlled by the sequence-specific recognition of a *cis*-acting replicator by ORC and associated initiator proteins, but the fission yeast replicators are larger than those of its budding yeast counterpart (18, 19, 50, 66, 68, 69). Initiation activity of yeast ARS elements in chromosomal DNA depends not only on specific DNA sequences in the ARS but also on chromatin structure and chromosomal position (13, 34, 36, 51, 63, 67, 70, 79, 88).

Extensive mapping of replication start sites in mammalian chromosomes has revealed that replication at most but not all loci begins at a few high-frequency start sites contained within a broad zone of initiation (4, 37, 59, 62, 82, 83; see also studies reviewed in references 12, 24, and 40). One of the most thoroughly mapped high-frequency initiation regions (IRs) in mammalian chromosomes is the region downstream from the dihydrofolate reductase (DHFR) gene in Chinese hamster ovary (CHO) cells (Fig. 1A). This region contains a 55-kb zone of delocalized origin activity containing three preferred start sites: origin beta (ori-β), centered approximately 17 kb downstream from the DHFR gene; ori-β′ just downstream from ori-β; and ori-γ, located 23 kb further downstream (5, 14, 42, 43, 44, 52, 55, 57, 71, 85, 86, 89).

Despite the progress in mapping initiation sites, the specific genetic elements necessary to direct initiation of mammalian DNA replication remain poorly understood. The existence of preferred IRs raises the question of whether specific DNA sequences within or neighboring an IR may direct initiation of replication at the IR or even in a broad zone more distant from the preferred IR. Several lines of evidence are consistent with the notion that sequences neighboring a preferred IR may constitute a mammalian replicator element. In the human lamin B2 IR, DNase I footprinting has revealed that a specific sequence is protected in a cell cycle-dependent manner, suggesting that it may be a binding site for an initiator protein complex (1, 29, 39). Moreover, replication at the lamin B2 origin was shown to initiate within a 3-bp sequence that overlaps the footprint region (2). A site hypersensitive to micrococcal nuclease has been mapped in the DHFR ori-β IR locus (72), and there is preliminary evidence that hamster ORC2, a subunit of the ORC complex, binds within the DHFR ori-β IR (cited in reference 12). Recent genetic analysis of the human β-globin (4) and the human c-*myc* (62) IRs at an ectopic chromosomal locus demonstrated that a defined sequence of 2 to 8 kb can be sufficient to direct initiation in the ectopic locus. Furthermore, the putative c-*myc* replicator even induced new start sites in the flanking chromosomal DNA (62). Taken together, these studies suggest that initiation of chromosomal DNA replication in mammalian cells may be directed by spe-

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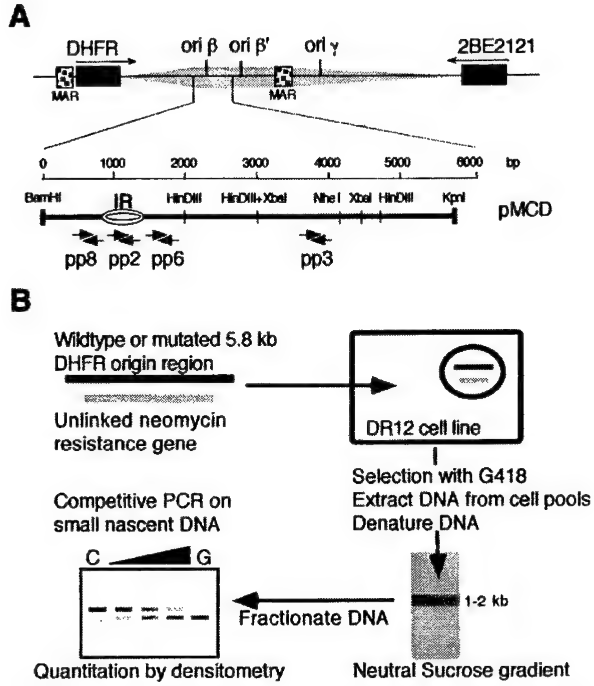

FIG. 1. (A) Features of the endogenous DHFR ori-β IR. Preferred start sites of DNA replication—the ori-β, ori-β′, and ori-γ sites; a 55-kb initiation zone (shaded area) between the DHFR- and 2BE2121-coding sequences; and matrix-attached regions (MARs, stippled boxes)—are indicated (5, 14, 42, 43, 44, 52, 55, 57, 71, 85, 86, 89). The 5.8-kb fragment of the DHFR ori-β region (pMCD), extending from the *Bam*HI to the *Kpn*I recognition sequences, is indicated. Arrows denote the positions of the primers used in the competitive PCR assay for origin function and correspond to primer pairs used in previous DHFR ori-β mapping studies (71). (B) Strategy for quantitating initiation occurring in exogenous DHFR ori-β fragments in ectopic chromosomal locations. A 5.8-kb wild-type or mutated DHFR ori-β fragment was coelectroporated with a neomycin resistance gene into the DR12 cell line, a CHO derivative containing a 150-kb deletion encompassing the entire DHFR locus (47). After selection with G418, total DNA was isolated, heat denatured, and size fractionated on a 5 to 30% linear neutral sucrose gradient. The fraction containing single-stranded DNA with a length of 1 to 2 kb, representing nascent DNA, was isolated, and the abundance of ori-β target sequences contained in the fraction was quantitated by competitive PCR.

cific *cis*-acting DNA sequence elements that constitute a replicator similar to those characterized in yeasts.

The complex organization of the DHFR initiation zone raises the question of whether it differs structurally from the putative replicators containing the c-*myc*, lamin B2, and β-globin IRs. For example, the cluster of preferred IRs may represent a novel replicator organization with multiple elements dispersed throughout the 55-kb initiation zone that are interdependent and cannot function independently as replicators. Alternatively, the three preferred start sites, ori-β, ori-β′, and ori-γ, within the delocalized initiation zone could represent separable but redundant genetic elements that ensure the faithful replication of this locus (72). In this latter case, the regions surrounding each of the three start sites might serve as individual replicators able to direct initiation when placed at an ectopic chromosomal location.

To address the question of whether a defined DNA sequence encompassing DHFR ori-β can direct initiation of DNA replication in chromosomal DNA, we have generated stably transfected cells containing the exogenous ori-β IR in random ectopic chromosomal locations and measured ori-β initiation activity by using a competitive PCR-based nascent strand abundance assay. The results presented here demonstrate that a 5.8-kb sequence containing the ori-β IR is sufficient to direct initiation in multiple ectopic chromosomal sites and that specific DNA sequences within and flanking the IR are essential for efficient initiation activity at the DHFR ori-β region.

MATERIALS AND METHODS

Cell culture. Diploid Chinese hamster ovary K1 (CHOK1) cells and DR12 cells—CHOK1 derivatives containing 150-kb deletions of the entire DHFR locus (47)—were grown in Ham's F12 medium supplemented with 10% fetal calf serum (Life Technologies, Rockville, Md.) at 37°C and 4% CO_2.

Plasmid constructs. The plasmid pMCD, containing the 5.8-kb *Bam*HI-*Kpn*I fragment, nucleotides (nts) 1 through 5793 (GenBank accession no. Y09885) of the DHFR ori-β locus in the vector pUC19 (90), was the kind gift of N. H. Heintz (16). Deletion mutant pAKO was generated by digestion of pMCD with *Apa*I (nt position 1127), removal of overhanging ends using T4 DNA polymerase, and religation. Mutant pMCDΔAT was created by partial digestion of pMCD with *Sph*I (nt position 2163), followed by complete digestion with *Eco*RV (nt position 2507), releasing a 344-bp fragment; the other fragment was then blunt ended and religated. The mutant pMCDΔDNR was created by partial digestion of pMCD with *Xba*I (nt position 4454) followed by complete digestion with *Nhe*I (nt position 4219), releasing a 235-bp fragment; the remaining fragment was blunt ended and religated. Mutant pMCDΔTR was created by using PCR and mutagenic primers (5′GACAAAAACAATCGATAAATAAG and 5′CTTATTTAT CGATTGTTTTTGTC) to insert a *Cla*I restriction site at nt position 686, relative to the *Bam*HI site. The resulting plasmid was partially digested with *Pvu*II (nt position 949), followed by complete digestion with *Cla*I, releasing a 263-bp fragment; the remaining fragment was blunt ended and religated. The pSV2neo plasmid contains a full-length neomycin resistance gene (Clontech Laboratories, Inc., Palo Alto, Calif.). Plasmid K126 contains the 3-kb *Bam*HI-*Xba*I fragment of the DHFR ori-β region in the vector pBluescript. Plasmid Mut8-2 contains the *Sac*I/*Acc*I fragment of the DHFR ori-β region in the vector pBluescript.

Transfection. The *Bam*HI-*Kpn*I ori-β fragment cloned in pUC19 was linearized with *Aat*II to generate the 5.8-kb DHFR insert flanked by 1,271 and 1,401 bp of vector DNA. Four micrograms of the digest mixed in a 3:1 molar ratio with *Pvu*I-linearized pSV2neo DNA was electroporated into 5×10^6 DR12 cells using a BioRad Gene Pulser at 360 V and 600 μF. Cells were plated in 75-cm² tissue culture flasks. After 24 h, the medium was replaced with Ham's F12 supplemented with 10% fetal calf serum and 0.5 mg of G418 (Life Technologies) per ml. After 4 weeks of growth under selection, exponentially proliferating cells from four 150-cm² flasks, grown to 70% confluency, were harvested, and total DNA was isolated and analyzed as described below. Under these transfection conditions, the average number of copies of exogenous DHFR ori-β integrated per cell in pools of cells was highly reproducible (see below). Single cell clones were isolated through serial dilution into a 96-well plate and expanded under drug selection for 14 weeks to four 150-cm² flasks. Isolation of total genomic DNA and its analysis were carried out as described below for pools of transfected cells.

Quantitation of stably transfected DNA. The integration of ectopic DHFR ori-β fragments into DR12 genomic DNA was monitored by PCR analysis of DNA from transfected cells. PCR amplification was carried out with primer set 2 (Table 1) and with 5 ng of total genomic DNA, isolated from transfected DR12 cells or from CHOK1 cells, in a Perkin-Elmer 4800 thermal cycler (35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s). Amplification products were resolved by 7% polyacrylamide gel electrophoresis (PAGE), stained with ethidium bromide, and quantified by densitometry (IPLab Gel software; Signal Analytics Corp., Vienna, Va.). The ratio of the exogenous DHFR ori-β products to the endogenous CHOK1 ori-β products, termed the integration ratio, was consistently close to 1 after 4 weeks of drug selection. Thus, although the integration ratio is not used to calculate the initiation activity, the copy number

TABLE 1. Oligonucleotide primer sequences and positions relative to the *Bam*HI site of pMCD

Primer	Sequence 5′–3′	Position[a]
8SX	CTCTCTCATAGTTCTCAGGC	470–489
8DX	GTCCTCGGTATTAGTTCTCC	651–670
2SX	GTCCCTGCCTCAAAACACAA	1070–1089
2DX	CCTTCATGCTGACATTTGTC	1329–1348
6SX	AACTGGCTTCCCAAGAAATT	1517–1536
6DX	AACCTCTGAACTGTAAGCTG	1666–1685
3SX	GGACACTAAGTCTAGGTACTACA	3882–3904
3DX	GCTGGGATAAGTTGAAATCC	4121–4140
8SXCOM[b]	GTCGACGGATCCCTGCAGGTCATTCATCAAGCTGGAAAGC	529–548
8DXCOM	ACCTGCAGGGATCCGTCGACTCCATGGCAGTCTTCACACT	549–569
2SXCOM	GTCGACGGATCCCTGCAGGTAAGGAAGGAAAGAAAGGGCCC	1126–1146
2DXCOM	ACCTGCAGGGATCCGTCGACCTCAGTGAGTCCACTTGCTTT	1147–1168
6SXCOM	GTCGACGGATCCCTGCAGGTATAGAAACCCCAGCTAAGAC	1587–1606
6DXCOM	ACCTGCAGGGATCCGTCGACTGCTGTGAAGAGACACCATG	1607–1626
3SXCOM	GTCGACGGATCCCTGCAGGTTAGGAAACTGAGATGCCAGG	3992–4010
3DXCOM	ACCTGCAGGGATCCGTCGACAGGACTCAGCTCTTACTAAC	4011–4031

[a] nt position relative to the *Bam*HI recognition sequence in the DHFR ori-β fragment pMCD, defined as position 1 (GenBank accession no. Y09885).
[b] Underlined sequence is the 5′ tail of the PCR primer which gave rise to the 20-bp insertion in the competitor plasmids (see Materials and Methods for construction).

of integrated pMCD fragments in DR12 cells, on average, mimics the copy number of the endogenous DHFR ori-β in CHOK1 cells. In some experiments, the PCR analysis was carried out in parallel with primer pairs 2 and 3; the resulting ratios did not differ significantly.

The structure of the integrated DHFR ori-β fragment was confirmed through PCR analysis of the integrated DHFR ori-β fragment using six sets of PCR primers (FullF [5′-GCTATGACCATGATTACGCC] and 8DX, 8SX and 2DX, 2SX and 6DX, 6SX and 3ATR [5′-CAGGCCAGTGTTTAGATGCTGG], 3ATF [5′-GGGATTAAAGGCATGCACCACC] and 3DX, and 3SX and FullR [5′-GGTTTTCCCAGTCACGACG]) on 20 ng of pMCD plasmid DNA or 1 μl of the largest DNA fraction from the sucrose gradient containing pMCD-transfected DR12 cells. PCR amplification was carried out in a Perkin-Elmer 4800 thermal cycler (50 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 5 min). Amplification products were resolved by 7% PAGE, stained with ethidium bromide, and visualized under UV light.

DNA isolation and gradient centrifugation. Total genomic DNA was isolated from harvested cells according to the manufacturer's instructions (Nucleon II; Scotlabs). Five-hundred micrograms of resuspended total genomic DNA in 1 ml of TE (10 mM Tris-HCl [pH 8.0]–1 mM EDTA) was heat denatured at 100°C for 10 min, followed by a 10-min incubation in ice-water. DNA was loaded onto a 5 to 30% linear neutral sucrose gradient and centrifuged at 26,000 rpm (Sorvall AH-629 rotor) for 17 h at 20°C. Fractions of 1 ml each were collected, and the fraction enriched in nascent single-stranded DNA (ca. 1 to 2 kb in length) was dialyzed against TE and used as a template for PCR amplification. As size markers, 50 μg of pMUT8-2 digested with *Kpn*I and *Nde*I to produce fragment sizes of 921 and 2,972 bp and 50 μg of pK126 digested with *Stu*I and *Bgl*II to produce fragment sizes of 1,636 and 4,338 bp were loaded on a sucrose gradient and run concurrently with each set of experiments. A 50-μl aliquot of each marker fraction was subjected to electrophoresis on a 1% agarose gel at 200 mA for 2 h, and DNA was visualized by ethidium bromide staining. For control PCR, total genomic DNA was sheared to fragments 1 to 2 kb in size by sonication for 15 s at 25% power (250/450 Sonifier; Branson Ultrasonics Corp., Danbury, Conn.), heat denatured, and fractionated on a 5 to 30% linear neutral sucrose gradient as described above.

Construction of PCR competitive templates. Competitors were constructed essentially as previously described (71). Briefly, PCR amplification was performed with pMCD template using the standard primer sets 8, 2, 6, and 3 and their corresponding competitor primer sets, as denoted by the suffix COM, which contain 20-bp insertions (Table 1). These PCR products were used as templates for another round of PCR amplification with the standard primer sets, resulting in PCR amplification products which were identical to their pMCD target sequence except for the 20-bp insertion. The mutated PCR amplification products were cloned into pMCD to create competitive templates for the PCR-based nascent strand abundance assay.

PCR-based nascent strand abundance assay. The competitive PCR nascent strand abundance assay was performed using primer sets 8, 2, 6, and 3 (31, 53, 71; Table 1) essentially as previously described. Each PCR mixture included the corresponding competitor plasmid DNA, which had been precalibrated against 20 ng of total genomic DNA from asynchronously growing CHOK1 cells. Assuming 3×10^9 bp per haploid genome, 20 ng of genomic DNA would correspond to 6,000 molecules of competitor (31). Amplification reactions with each primer set contained increasing amounts of the size-fractionated nascent DNA and the precalibrated amount of the corresponding competitor DNA. PCR was carried out in a Perkin-Elmer 4800 thermal cycler (50 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s). Similar results were obtained using 35 cycles with the same program (data not shown), consistent with the expectation that competitive PCR should be independent of cycle number (31). Amplification products were resolved by 7% PAGE, stained with ethidium bromide, and quantified by densitometry (IPLab Gel software; Signal Analytics Corp.). The intensity of the signal was linearly proportional to the amount of stained DNA under the conditions used.

The ratio of PCR amplification products from nascent genomic template relative to the PCR amplification products from the competitive template (nascent/competitor) was plotted on the x axis against the volume of input nascent genomic DNA template on the y axis. Linear regression analyses were performed on the data (KaleidaGraph software; Synergy Software, Reading, Pa.), resulting in correlation coefficients greater than 0.97. In order for the calculations to be accurate, the correlation coefficient must be at least 0.97, and the equivalence point must be contained within the volumes used in the PCR analysis. The slope of this linear regression, along with the known molecules of calibrated competitor per microliter, was used to calculate the molecules of nascent DNA per microliter for each primer set. Since the number of base pairs in each amplification product is known, the grams per mole of target DNA can be calculated using the conversion of 660 g/mol of base pairs. By converting the molecules of nascent DNA target per microliter for each primer set to moles per microliter using Avogadro's number and multiplying this by the grams of target DNA per mole, the DNA concentration of nascent DNA target for each primer pair can be calculated. The concentration of target molecules for each primer pair can then be expressed relative to the concentration of total DNA in the nascent fraction, as determined by absorbance at 260 nm (termed abundance). To facilitate comparison between separate experiments, the abundance of target sequences for each primer pair was expressed relative to the abundance of the target sequences for the distal pp3 in the same experiment (defined as 1), and this ratio was termed initiation activity.

RESULTS

A DHFR ori-β fragment functions efficiently as an origin in ectopic chromosomal locations. Given that DNA fragments containing the β-globin (4) and the c-*myc* (62) IRs supported efficient DNA replication at ectopic chromosomal locations, we asked whether a small DNA fragment containing DHFR ori-β and flanking DNA but lacking the downstream ori-β′ and ori-γ regions would direct initiation of DNA replication when

located at ectopic chromosomal sites. To address this question, we chose the experimental strategy diagramed in Fig. 1B. A 5.8-kb fragment containing the ori-β IR (pMCD [16]) but not the ori-β′, ori-γ, or matrix attachment regions or the DHFR coding sequences was cotransfected by electroporation with an unlinked neomycin resistance marker into DR12 cells, a CHOK1-derived line lacking both DHFR loci (47). We chose an unlinked resistance marker to ensure that any initiation of DNA replication occurring within the ori-β fragment would not be influenced by transcription of the neomycin gene in the same construct. To facilitate the stable integration of the DNA fragments into the chromosomal DNA, plasmids containing the ori-β DNA fragment were first linearized in the vector portion by restriction endonuclease digestion. Under these conditions, integration of exogenous DNA fragments has been reported to be random and without significant loss of DNA sequences at the ends of the fragment (35, 77).

As a strategy to minimize potential position effects of the integration site, nascent DNA was isolated from pools of uncloned transfectants. We reasoned that if position effects caused by flanking DNA at the integration sites of the ectopic chromosomal ori-β fragments occurred, they might be masked in pools of uncloned cells, thereby enhancing the reproducibility of the assay. Total DNA was heat denatured and size fractionated by centrifugation. The fraction containing single-stranded DNA (ca. 1 to 2 kb in length) was used as a template for PCR amplification in competitive PCR-based nascent strand abundance assays with four sets of primers (Fig. 1A). Three of the selected primer sets (pp8, pp2, and pp6) were located within and directly flanking the IR, as determined by previous mapping studies (52, 57, 71). A fourth primer set (pp3), located distal to the IR and outside of the 1- to 2-kb nascent DNA strand template, was used to normalize the data to an outlying non-origin primer set and facilitate comparison between separate experiments. Since the 5.8-kb DNA fragment integrates randomly into the genome, it is important to normalize to a primer set contained within the fragment. In this way, if the initiation activity of the construct is affected by neighboring chromatin, then the entire fragment will be affected, including all primer sites. Hence, the initiation activity of the ectopic IR was expressed as the ratio of the IR target sequences over the non-IR sequence in the 1- to 2-kb single-stranded DNA fraction.

To first validate our competitive PCR-based nascent strand abundance assay, we used it to confirm the preferential initiation at the endogenous DHFR ori-β region in CHOK1 cells. DNA sequences that are close to the ori-β IR are expected to be enriched in the short single-stranded DNA fraction, compared to more distal DNA sequences which are expected to be represented only in longer nascent DNA strands. Consistent with previous studies (52, 57, 71), the amount of nascent DNA template required to amplify an amount of target DNA equivalent to the precalibrated competitor DNA with primer pair 2 (pp2), centered over the previously mapped ori-β IR, was smaller than with primer pairs 8, 6, and 3, which were more distant from the IR (Fig. 2A). To better illustrate the differences among the 4 sets of primers in amplification of the genomic target, the gels shown in Fig. 2A are from a single experiment using equal volumes of the nascent DNA template fraction, except for a 1:10 dilution of the fraction used for pp2. When the equivalence point was not reached within the tested volumes of the nascent DNA fraction, the experiment was repeated using either increased or decreased amounts of the nascent fraction until the equivalence point was included (data not shown). Quantitative evaluation of one such experiment is shown in Table 2. The abundance of pp2 target DNA sequences was more than 10-fold higher than the abundance of target sequences for the outlying pp3 in the nascent CHOK1 DNA fraction. To compare the abundance of pp2 target sequence in nascent CHOK1 DNA among multiple independent experiments, this abundance was expressed relative to the abundance of pp3 sequences in each experiment to give initiation activity. The average initiation activity from five independent experiments with CHOK1 cells is shown in Fig. 2C (black bars). Consistent with previous studies (52, 57, 71), the initiation activity centered over the previously mapped IR in the endogenous DHFR ori-β region was strongly enhanced compared to that in the flanking sequences (Fig. 2C, black bars).

To establish a baseline or background value for comparison with nascent strand abundance data, the experiment was repeated under identical conditions, except that total CHOK1 DNA, isolated from asynchronously growing cells, was sheared to 1- to 2-kb fragments, heat denatured, and size fractionated. The fraction containing the sheared, single-stranded DNA of 1 to 2 kb in size was used as the template in competitive PCR assays. In total genomic DNA, the target sequences for each primer pair should be essentially equally represented, and thus, the amounts of amplification product generated with each primer pair should be similar. Indeed, the amounts of amplified DNA were similar with three of the four primer pairs and somewhat reduced for pp8 compared to the other primer pairs (Fig. 2B; Table 2). Comparison of these results with the enhanced abundance of pp2, pp6, and pp8 target sequences in the nascent DNA fraction from asynchronous CHOK1 cells indicated that the peak of initiation activity depended on the enrichment for nascent DNA template and did not arise through differences between primers in amplification efficiency or calibration error (Fig. 2C, compare black and white bars). Thus, the abundance of target sequences generated by amplification of the sheared-DNA control was considered to represent the empirical background activity of the assay system.

The PCR-based nascent strand abundance assay was then used to measure initiation activity at ectopic ori-β sequences in genomic DNA from pools of stably transfected DR12 cells. The transfection conditions were chosen so that the average copy number of ectopic ori-β fragments per cell in the pool of cells would mimic that of the endogenous ori-β in CHOK1 DNA. To confirm this, total genomic DNA isolated from uncloned drug-resistant pools of DR12 cells was used as a template for PCR amplification. For comparison, an equal amount of total genomic DNA isolated from CHOK1 cells was used as a template in a parallel amplification. The amplification products of both reactions were visualized by PAGE and ethidium bromide staining. Amounts of amplification product obtained with both samples were quantitated and found to be very similar, implying that the average copy number of ori-β per transfected cell in the pool was close to that of the endogenous ori-β in CHOK1 cells (Fig. 3A). However, it should be noted that the determination of the initiation activity of the trans-

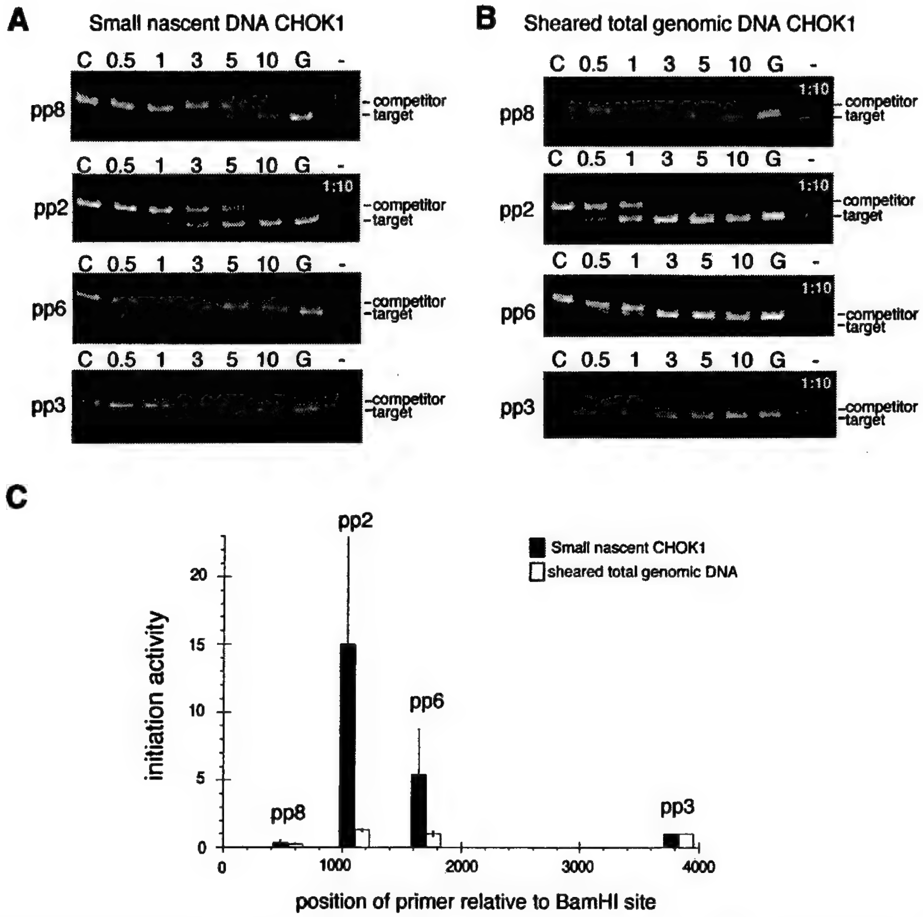

FIG. 2. Initiation of DNA replication at the endogenous DHFR ori-β site in asynchronous CHOK1 cells. (A) PCR amplifications were performed with each of the four primer pairs and with size-fractionated nascent DNA template from asynchronous CHOK1 cells in the presence of a precalibrated amount of the corresponding competitor DNA. Amplification products were analyzed by PAGE and ethidium bromide staining. Control lanes: C, competitor template only; G, nascent genomic DNA template only; −, no template. Numbers above each lane represent the volume in microliters of nascent DNA added to the PCR mixture. Amplification reactions with pp2 used a 1:10 dilution of the nascent DNA. (B) PCRs were performed and analyzed as in panel A, except that the template was a 1:10 dilution of sheared, denatured total genomic CHOK1 DNA 1 to 2 kb in length. (C) Amplification products generated with either nascent DNA from asynchronous CHOK1 cells (black bars) or sheared total genomic DNA from asynchronous CHOK1 cells (white bars) were quantitatively evaluated for five independent experiments with each type of template. As a measure of initiation activity, the abundance of each target sequence in nascent genomic DNA was normalized to the abundance of pp3 target sequences in the corresponding experiment, which was set equal to 1 (see example in Table 2), and the average of five experiments is shown. Bars indicate the standard error of the mean (SEM).

fected DHFR ori-β fragments does not depend on this approximate copy number (see Materials and Methods).

To confirm that the structure of the integrated DHFR ori-β fragments was not rearranged or truncated, large single-stranded genomic DNA isolated from uncloned drug-resistant pools of pMCD-transfected DR12 cells was used as a template for PCR amplification. PCR amplification was carried out with a series of primer sets which would result in overlapping am-

TABLE 2. Abundance of DHFR ori-β target sequences in nascent DNA[a]

DNA source	Abundance[b] of DHFR ori-β target sequences (initiation activity[c]) with primer pair:			
	pp8	pp2	pp6	pp3
CHOK1	8.49×10^{-8} (0.4)	2.80×10^{-6} (12.8)	3.65×10^{-7} (1.7)	2.18×10^{-7} (1.0)
pMCD pool	1.32×10^{-7} (1.3)	2.59×10^{-6} (25.4)	6.63×10^{-7} (6.5)	1.02×10^{-7} (1.0)
Total sheared DNA	3.45×10^{-7} (0.3)	1.37×10^{-6} (1.3)	1.03×10^{-6} (1.0)	1.06×10^{-6} (1.0)

[a] Values in the table are from a typical experiment.
[b] Abundance was calculated as described in Materials and Methods.
[c] Initiation activity was calculated as described in Materials and Methods.

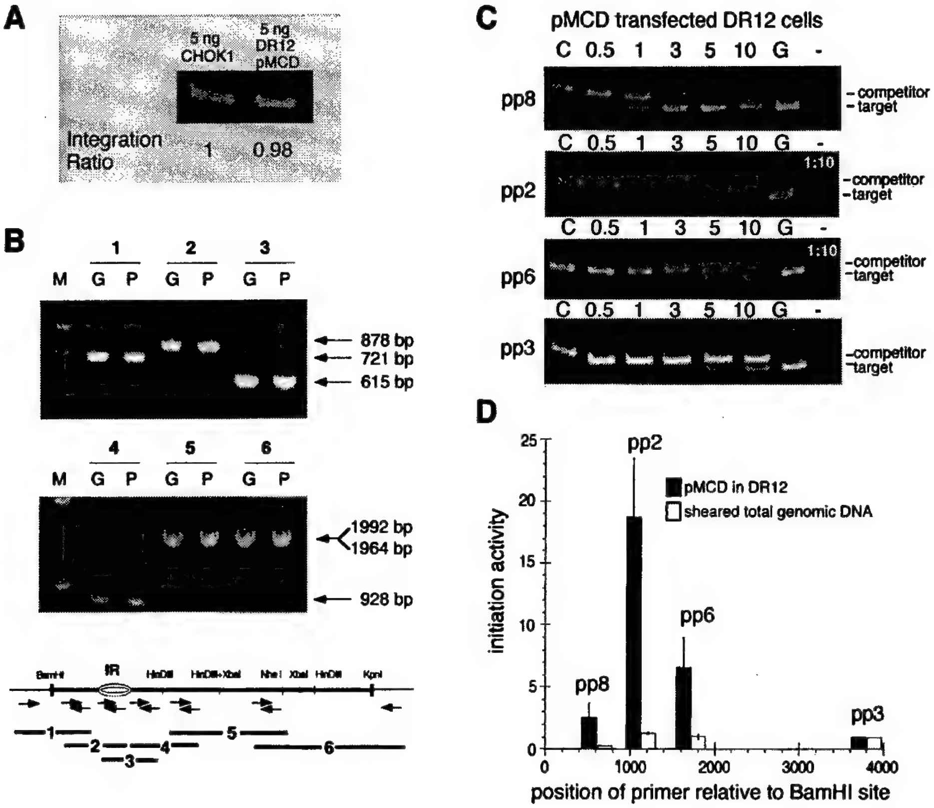

FIG. 3. Initiation of DNA replication in the exogenous 5.8-kb ori-β fragment in DR12 cells. (A) The integrated exogenous DHFR ori-β fragment in 5 ng of DNA from uncloned pools of DR12 cells and the endogenous ori-β in 5 ng of CHOK1 DNA were amplified by PCR. The ratio of the amplification products in DR12 relative to those of endogenous ori-β in CHOK1 is indicated, and this ratio suggests that the copy number of exogenous ori-β fragments present in the pool of transfectants mimics that of the endogenous locus in CHOK1. (B) The structure of the integrated DHFR ori-β fragments in DR12 pools was determined by PCR amplification of either pMCD plasmid DNA (lanes P) or large single-stranded genomic DNA isolated from uncloned drug-resistant pools of pMCD-transfected DR12 cells (lanes G). Amplification reactions were performed with a panel of six primer sets spanning the 5.8-kb DHFR ori-β fragment and the flanking vector sequences (bottom). The resulting overlapping PCR amplification products, labeled 1 through 6, were analyzed by PAGE and ethidium bromide staining. M indicates a DNA size marker. The sizes of the resulting PCR amplification products are indicated. (C) PCR amplifications were performed with each of the four primer pairs and size-fractionated nascent DNA from asynchronous pMCD-transfected DR12 cells in the presence of a precalibrated amount of the corresponding competitor DNA. Amplification products were analyzed by PAGE and ethidium bromide staining. Control lanes: C, competitor template only; G, nascent genomic DNA template only; –, no template. Numbers above each lane represent the volume in microliters of nascent DNA added to the PCR mixture. Amplification reactions for pp2 and pp6 used a 1:10 dilution of the nascent DNA. (D) Amplification products generated with either nascent DNA from a pool of asynchronous pMCD-transfected DR12 cells (black bars) or sheared total genomic DNA from asynchronous pMCD-transfected DR12 cells (white bars) were quantitatively evaluated for seven independent transfection experiments. As a measure of initiation activity, the abundance of each target sequence in nascent genomic DNA was normalized to the abundance of pp3 target sequences in the corresponding experiment, which was set equal to 1 (see example in Table 2), and the average of the seven experiments is shown. Bars indicate the SEM.

plification products, extending from the flanking vector sequences on one side of the DHFR insert through the insert and into the flanking vector sequences on the other side of the insert (Fig. 3B). For comparison, pMCD plasmid DNA was used as a template in a parallel amplification. The amplification products of both reactions were visualized by PAGE and ethidium bromide staining. As seen in Fig. 3B, the amplification products generated from the transfected DNA (lanes G) were identical to those generated from the plasmid (lanes P). This result demonstrates that most of the integrated DNA fragments in DR12 pools were intact and had not undergone rearrangement during integration into the genome.

The abundance of ori-β target sequences in nascent DNA originating from the ectopic pMCD fragment in pools of transfected DR12 cells was then quantitated by competitive PCR. As seen in Fig. 3C, the amount of nascent DNA needed to amplify an amount of target DNA equivalent to the precalibrated competitor DNA with primer pair 2 was 10-fold less than with the flanking pp8 and the distal pp3, and severalfold less than with the flanking pp6. The abundance of target DNA for each primer pair in the nascent DNA fraction of the transfected cell pool was similar to that of the corresponding target DNA in nascent DNA from CHOK1 cells, as shown in the example in Table 2. Combining data from seven independent

TABLE 3. Abundance of DHFR ori-β target DNA sequences in nascent DNA[a]

DNA source	Copy no.[d]	Abundance[b] of DHFR ori-β target DNA (initiation activity[c]) with primer pair:			
		pp8	pp2	pp6	pp3
Clone 1	1	2.04×10^{-7} (0.6)	2.49×10^{-6} (6.9)	1.41×10^{-7} (0.4)	3.62×10^{-7} (1.0)
Clone 2	0.7	6.16×10^{-8} (0.9)	5.03×10^{-7} (7.4)	7.73×10^{-8} (1.1)	6.79×10^{-8} (1.0)
Clone 3	0.9	8.11×10^{-8} (0.4)	7.05×10^{-7} (3.5)	7.09×10^{-8} (0.4)	2.04×10^{-7} (1.0)
Clone 4	0.8	4.07×10^{-8} (0.4)	6.04×10^{-7} (5.8)	5.81×10^{-8} (0.5)	1.05×10^{-7} (1.0)
Clone 5	1	3.03×10^{-8} (0.4)	6.87×10^{-7} (9.4)	8.50×10^{-8} (1.2)	7.31×10^{-8} (1.0)
Clone 6	0.7	8.77×10^{-8} (0.6)	3.88×10^{-7} (2.7)	7.36×10^{-8} (0.5)	1.45×10^{-7} (1.0)

[a] Values in the table are from a typical experiment with each clone.
[b] Abundance was calculated as described in Materials and Methods.
[c] Initiation activity was calculated as described in Materials and Methods.
[d] Integration ratio of ori-β fragment relative to endogenous ori-β in CHOK1 cells.

transfection experiments revealed that the target sequence for pp2 was significantly more abundant in the nascent DNA fraction than flanking target sequences, as would be expected for an active origin of DNA replication (Fig. 3D, black bars). The initiation activity of the ectopic ori-β IR was enhanced more than 10-fold relative to that in the distal pp3 sequences, closely resembling the results obtained with endogenous ori-β in CHOK1 cells (compare Fig. 3D and Fig. 2C). These results suggest that the 5.8-kb fragment of the DHFR ori-β region is sufficient to direct initiation in ectopic chromosomal locations and that the ectopic ori-β fragment functions with an efficiency comparable to that in the endogenous locus.

Ori-β functions in multiple ectopic locations, but exhibits some position effects. Since chromosomal context is an important determinant of origin function in mammalian cells (3, 17, 27, 30, 33, 54, 56), the function of the exogenous ori-β IR may be sensitive to position effects that were not detected in the uncloned cell pools. If initiation activity of the 5.8-kb fragment is affected by chromosomal context, one might expect to find that initiation activity of the ectopic ori-β region would vary among clonal cell lines with different ori-β integration sites. To test for variability in initiation activity, six individual cell lines were cloned from a resistant-cell pool. To estimate the amount of integrated exogenous ori-β fragment in each clone, total genomic DNA isolated from each cell line was used as a template for PCR amplification with pp2. For comparison with the endogenous ori-β region, an equal amount of total genomic DNA isolated from CHOK1 cells was used as a template in a parallel amplification. The amplification of the exogenous ori-β DNA fragments differed slightly from clone to clone. Clones 1, 3, and 5 contained about the same copy number found for the endogenous ori-β region in CHOK1 DNA (Table 3). Clones 2, 4, and 6 had 20 to 30% less ori-β fragment, possibly suggesting some loss of ori-β sequences during expansion of the cloned cell lines.

The abundance of the exogenous ori-β IR target sequences in the nascent DNA fraction was quantitated for each cell clone, as shown in the examples in Table 3. For each cell clone, the abundance of target DNA for pp2 was higher than the abundance of target DNA for the flanking primer sets (Table 3). The relative initiation activity of the clones varied from 2.7- to 9.4-fold higher than in the distal pp3 sequences. Thus, the initiation activity of the clones ranged from just over background in two of the clones to about half the activity observed with uncloned pools of cells (compare Table 3 and Fig. 3D). The strong activity observed for the pMCD-transfected cell pools in repeated experiments (Fig. 3D) suggests that the variability from one clone to another was probably masked when pools of transfected cells containing many different integration sites were tested. Although the ectopic ori-β region was less active in each of the clones than in a typical experiment with an uncloned pool of transfectants (compare Tables 2 and 3), these results suggest that the exogenous 5.8-kb ori-β fragment was functional to some degree in six ectopic locations.

Specific *cis*-acting sequences are required for efficient DHFR ori-β initiation activity. To assess whether specific DNA sequences within the 5.8-kb fragment of the ori-β region were required for efficient initiation at the preferred IR, several ori-β deletion mutants were constructed (Fig. 4A). The regions chosen for deletion contain DNA sequences that could be functionally important in mammalian origins based on their resemblance to DNA elements identified in other origins as discussed below. To permit comparison of the initiation activity of mutated fragments with that of the parental pMCD ori-β fragment, mutations that would delete primer sites for PCR amplification were avoided.

The downstream end of the 5.8-kb DHFR fragment contains a region of GA dinucleotide repeats which has been shown to adopt non-B-form DNA (16). Such dinucleotide repeats have been postulated to slow or arrest replication fork progression in rodent cells (7, 74). To determine whether the GA repeat element affected ori-β initiation activity, we created a construct which deleted the GA repeat (pMCDΔDNR [Fig. 4A]).

The 5.8-kb ori-β DNA fragment contains a central AT-rich region (Fig. 4A) that was previously proposed to be a DUE (16). This region shares sequence homology with a cell cycle-dependent protein binding site in the human lamin B2 IR (1, 29, 39), suggesting it as a candidate for an initiator protein binding site (Fig. 4A, hatched box). Moreover, the AT-rich region has homology to the recently reported ORC-binding sequences in chorion gene amplification control element (ACE) ACE3 (6, 80) (Fig. 4A, checkered box). It also has homology with an AT-rich motif M found in *Schizosaccharomyces pombe* ARS elements (50). In order to determine whether the central AT-rich sequence in the DHFR ori-β region contributes to initiation activity, we deleted a 344-bp region containing this element from the 5.8-kb fragment (pMCDΔAT [Fig. 4A]).

The DHFR ori-β IR contains an AT-rich DNA sequence similar to ACE3 ORC binding sites (Fig. 4A, checkered box)

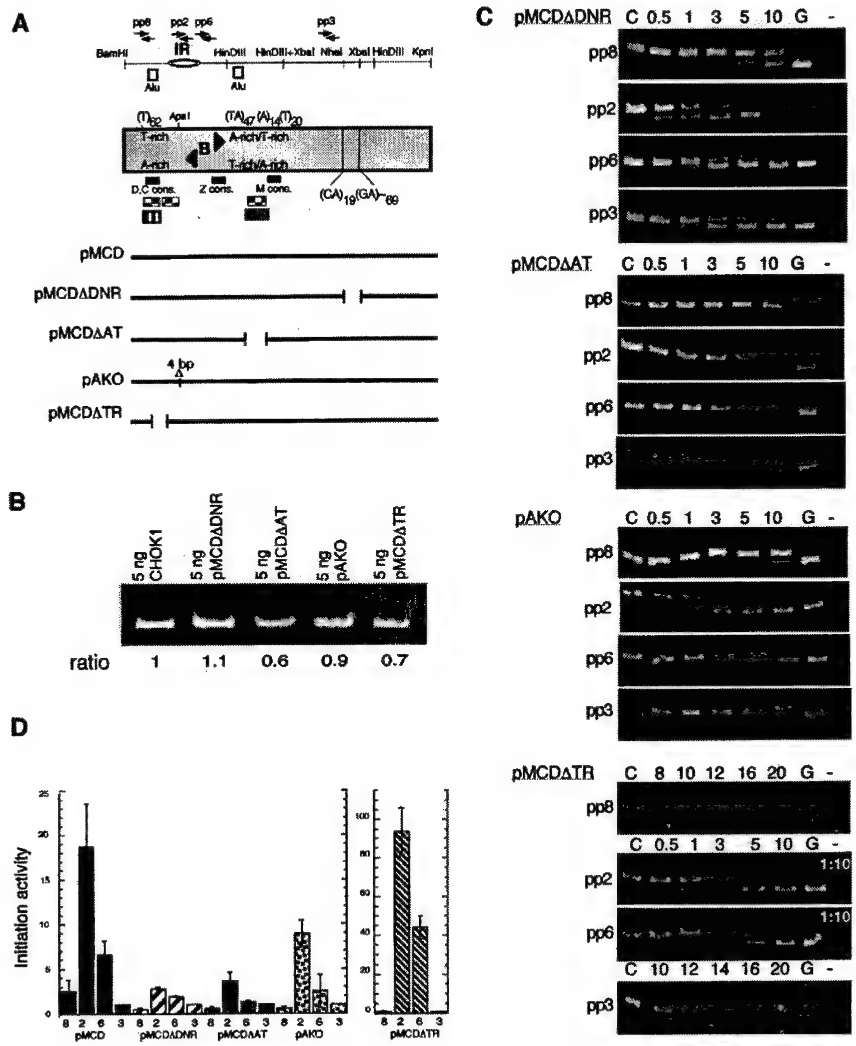

FIG. 4. Initiation of DNA replication in wild-type and mutant exogenous ori-β DNA fragments. (A) Unusual DNA sequences in the 5.8-kb ori-β fragment and the location of the deletion mutants are indicated on the restriction map of the region. Positions of *Alu*I repetitive elements (white boxes) and AT-rich sequences homologous to the *S. pombe* origin consensus motifs D, C, Z, and M (50) (small black boxes) are marked. AT-rich sequences homologous to a cell cycle-dependent DNase I genomic footprint in the human lamin B2 IR (1, 29, 39) (hatched box) and to the ORC-binding region in the *Drosophila* chorion ACE3 (6, 80) (checkered boxes) are noted. AT-rich sequences containing stably bent DNA (B) and binding sites for a zinc finger protein of unknown function, RIP60 (black arrowheads), are indicated (15, 16, 21, 45, 64). The position of a cell cycle-dependent nuclease-hypersensitive site (72) is indicated (striped box). (B) The integrated mutant DHFR ori-β fragments in 5 ng of DNA from uncloned pools of DR12 cells and the endogenous ori-β region in 5 ng of CHOK1 DNA were amplified by PCR and visualized by gel electrophoresis and ethidium bromide staining. The ratio of the mutant amplification products in DR12 relative to those of the endogenous ori-β region in CHOK1 is indicated and suggests that the copy number of the exogenous ori-β region present in the pool of transfectants mimics that of the endogenous locus in CHOK1. (C) PCR amplifications were performed with each of the four primer pairs and size-fractionated nascent DNA from asynchronous mutant-transfected DR12 cells in the presence of a precalibrated amount of the corresponding competitor DNA. Amplification products were analyzed by PAGE and ethidium bromide staining. Control lanes: C, competitor template only; G, nascent genomic DNA template only; −, no template. Numbers above each lane represent the volume in microliters of nascent DNA added to the PCR mixture; note that for pMCDΔTR, the nascent genomic DNA was used at a 1:10 dilution with pp2 and pp6. (D) The abundance of nascent DNA from pools of mutant-transfected DR12 cells from three independent transfection experiments was quantitatively evaluated. As a measure of initiation activity, the abundance of each target sequence in nascent genomic DNA was normalized to the abundance of pp3 target sequences in the corresponding experiment, which was set equal to 1 (see Table 4 for a typical experiment), and the average of three experiments with each mutant is shown. For comparison, the average initiation activity measured with nascent DNA from a pool of asynchronous wild-type pMCD-transfected DR12 cells (Fig. 3D) is also shown. Bars indicate the SEM.

TABLE 4. Abundance of DHFR ori-β target DNA sequences in nascent DNA[a]

DNA source	Abundance[b] of DHFR ori-β target DNA (initiation activity[c]) with primer pair:			
	pp8	pp2	pp6	pp3
pMCD pool	1.13×10^{-6} (3.7)	9.10×10^{-6} (29.6)	2.00×10^{-6} (6.5)	3.08×10^{-7} (1.0)
pMCDΔDNR pool	4.18×10^{-8} (0.3)	3.56×10^{-7} (2.5)	2.69×10^{-7} (1.9)	1.44×10^{-7} (1.0)
pMCDΔAT pool	1.98×10^{-7} (0.7)	1.18×10^{-6} (4.3)	4.45×10^{-7} (1.6)	2.73×10^{-7} (1.0)
pAKO pool	1.54×10^{-7} (0.4)	3.34×10^{-6} (7.6)	6.56×10^{-7} (1.5)	4.52×10^{-7} (1.0)
pMCDΔTR pool	1.26×10^{-7} (1.3)	1.20×10^{-5} (118.0)	4.66×10^{-6} (45.9)	1.01×10^{-7} (1.0)
Total DNA (pMCD pool)	1.60×10^{-6} (0.4)	5.60×10^{-6} (1.3)	3.37×10^{-6} (0.8)	4.18×10^{-6} (1.0)

[a] Values in the table are from a typical experiment.
[b] Abundance was calculated as described in Materials and Methods.
[c] Initiation activity was calculated as described in Materials and Methods.

(6, 12, 80). It also contains a stably bent DNA sequence and binding sites for the RIP60 protein (15, 16, 21, 45, 64). A deletion of 4 bp in a GC hexanucleotide between these elements was constructed to explore the effects of a small deletion on initiation activity (Fig. 4A, pAKO). Such minimal mutations in the ARS consensus sequence of budding yeast ARS elements have been shown to reduce or completely abolish ARS activity (58, 76, 84) by preventing ORC binding to the ARS (9, 32, 75, 78). A single-base-pair insertion between two T-antigen-binding sites in the simian virus 40 origin has also been shown to destabilize T-antigen binding by altering the spacing and consequently the interactions between the two T-antigen hexamers on DNA (20, 87).

Finally, a region upstream of the DHFR ori-β IR with an extensive T-rich stretch in one strand was chosen for deletion. Many characterized origins contain a sequence composed of a T-rich and an A-rich strand, the length of which appears to be critical for origin function, perhaps to facilitate strand separation (reviewed in reference 23). The T-rich stretch in the ori-β region contains a prominent cell cycle-dependent nuclease-hypersensitive site (72) (Fig. 4A, striped box) and an Alu repeat. Some Alu repeats have been correlated with DNA amplification and with autonomous replication activity of plasmid DNA (8, 48, 65). The T-rich region has sequence homology to the *Drosophila* ACE3 element (6, 80) (Fig. 4A, checkered box) and homology with AT-rich motifs C and D found in *S. pombe* ARS elements (50). In order to determine whether this T-rich region affects the initiation activity of the 5.8-kb fragment, we deleted a 263-bp region containing this element (pMCDΔTR [Fig. 4A]).

Mutant DNA fragments were transfected into DR12 cells, and drug-resistant pools of cells were selected. The amount of integrated exogenous ori-β fragment in each pool was monitored by PCR analysis of the genomic DNA as in Fig. 3A. Fig. 4B shows that the amount of exogenous ori-β in DNA from the mutant-transfected cell pools was about the same as the endogenous ori-β in an equal amount of CHOK1 DNA. Thus, the mutant origins were stably associated with chromosomal DNA in the same manner as the transfected wild-type 5.8-kb ori-β fragment (compare Fig. 4B and Fig. 3A).

The abundance of ori-β sequences in the small nascent DNA fraction prepared from pools of uncloned transfectants was then assayed by competitive PCR. A typical experiment with each mutant is shown in Fig. 4C and quantitatively evaluated in Table 4. The abundance of target DNA for pp2 in the nascent DNA fraction from three of the transfected mutants (pMCD ΔDNR, pMCDΔAT, and pAKO) was significantly lower than the abundance of pp2 target sequences in nascent DNA from the transfected wild-type ori-β region (Table 4). Comparing the abundance of pp2 target sequence in nascent DNA with that of flanking target sequences demonstrates that the initiation activity of these three deletion mutants was markedly reduced. In contrast, deletion of the upstream T-rich element (pMCDΔTR) resulted in a fourfold increase in the abundance of pp2 target sequence relative to the abundance of the flanking pp3 target sequence (Table 4), indicating that deletion of the upstream T-rich element enhanced initiation activity at the ectopic ori-β locus.

To facilitate comparison between the mutants, the abundance of target sequences for each primer pair in three separate transfection experiments with each mutant was normalized to the abundance of target sequences for pp3 in the same experiment. As seen in Fig. 4D, the initiation activity for pMCDΔDNR-transfected cell pools was strongly and reproducibly reduced compared to that in pMCD-transfected cell pools (two-tailed t test, P value = 0.01, representing a confidence level of 98%). Initiation activity of pMCDΔAT-transfected cell pools was also significantly reduced compared to wild-type-transfected pools (two-tailed t test, P value = 0.02, representing a confidence level of 96%). Indeed, the initiation activities with the pMCDΔDNR and pMCDΔAT mutants were only slightly above the empirical background for the assay as determined using sheared total DNA (Table 4; Fig. 3C, white boxes). Deletion of 4 bp within the IR (pAKO) significantly diminished the initiation activity, but the reduction was more modest (two-tailed t test, P value = 0.10, representing a confidence level of 80%). Deletion of the upstream T-rich element (pMCDΔTR) clearly stimulated initiation activity (two-tailed t test, P value = 0.01, representing a confidence level of 98%) compared to the wild-type activity. The results demonstrate that the sequences deleted in three of the mutants were critical for ori-β activity. In contrast, the T-rich region was dispensable for the initiation of DNA replication at the ori-β site and suggests that this region may suppress initiation activity of the 5.8-kb fragment.

DISCUSSION

A 5.8-kb region surrounding the DHFR ori-β IR functions as an independent chromosomal replicator. Evidence presented in this study indicates that a 5.8-kb fragment of the DHFR ori-β region placed in random ectopic chromosomal

locations was sufficient to direct initiation of DNA replication from the ori-β start site (Fig. 3C). These data were obtained by using a competitive PCR-based nascent strand abundance assay with nascent template DNA enriched only by heat denaturation and size selection. Thus, a possible concern is that the template fraction contained not only nascent DNA but probably also small DNA fragments generated by shearing in vitro and single-strand breaks in vivo. To validate the preparation methods and assays used to assess the initiation activity of the 5.8-kb ectopic ori-β fragment, we initially tested these procedures with the endogenous DHFR locus (Fig. 2C). Direct comparison of our data on nascent strand abundance at the ori-β site in CHOK1 cells with data obtained by using lambda exonuclease digestion to further enrich for RNA-primed nascent DNA after the size fractionation (38) reveals remarkable congruence (52). The ratio of nascent DNA at the center of the ori-β IR to flanking nonreplicating DNA in our study averaged about 15-fold in multiple experiments (Fig. 2C), quantitatively comparable to the 12-fold enrichment reported by Kobayashi et al. for an experiment with CHOK1 cells (compare Fig. 2C of this report with Fig. 6B in reference 52). The reproducibility of the results obtained with the two different methods of enrichment for nascent DNA in the endogenous ori-β locus in CHOK1 cells argues strongly that the application of our methods to analysis of the ectopic ori-β fragments in DR12 hamster cells accurately reflects the initiation activity emanating from the ectopic fragments. However, it should be noted that the variability in our experiments was somewhat greater than was observed with the additional enrichment for nascent DNA using lambda exonuclease (52).

The initiation activity of the exogenous ori-β region in uncloned pools of stably transfected cells was at least as great as that observed for the endogenous ori-β region in CHOK1 cells (compare Fig. 2C and Fig. 3D). This result indicates that the ori-β region does not require either ori-β′ or ori-γ to direct initiation of chromosomal DNA replication. Since the ectopic ori-β fragments were incorporated at random in the genome of the transfected cells, the exogenous DNA fragments could conceivably integrate into a chromosomal context that directed efficient initiation from the ori-β IR. However, this possibility seems unlikely since at least some initiation activity was detected at the ectopic ori-β IR in each individual cell clone tested (Table 3). A simpler interpretation is that the 5.8-kb fragment contains all of the sequences necessary to specify the start sites for replication at ori-β, and hence this fragment serves as an independent chromosomal replicator. Further support for this interpretation is provided by the demonstration that specific DNA sequences within the 5.8-kb fragment were necessary for efficient initiation at the ori-β start site (Fig. 4; Table 4).

Although the exogenous 5.8-kb ori-β fragment was functional to some degree in at least six random ectopic locations, the initiation activity of the ori-β fragment varied among the individual cell clones from just above background to about half of that detected in uncloned pools of transfectants (Table 3). This clonal variation may reflect position effects exerted by the flanking chromatin that were masked when ori-β activity was measured in uncloned pools of pMCD-transfected cells (Fig. 3D). The notion that ectopic ori-β was subject to position effects would be consistent with the lower initiation activity of the ectopic fragments in the cloned lines compared to that in uncloned pools of transfected cells. It should be noted that initiation activity of the ectopic ori-β fragment in the pools of transfectants was determined 4 to 5 weeks after transfection, whereas the initiation activity of the ori-β fragment in the subclones was determined 14 weeks after transfection due to the time required to expand the cloned cells. The decreased initiation activity of the subclones could thus be due to progressive chromatin-mediated repression of the integrated ori-β fragment over time. Gradual extinction of gene expression from stably transfected genes has been frequently observed after long-term propagation of transfected cells, but this extinction was avoided when the transfected genes were flanked by insulators (73). Similarly, *Drosophila* ACEs placed at ectopic sites have been shown to be subject to position effects that were prevented by flanking the ACE with insulators (60).

The possibility that the ectopic ori-β region may be subject to position effects raises the question of how the endogenous DHFR initiation zone escapes position effects and whether it may be protected by elements such as insulators. Interestingly, a 3.2-kb fragment at the 3′ end of the DHFR coding sequence in the endogenous locus appeared to be required for all replication activity in the 55-kb initiation zone (49). This 3.2-kb DNA fragment was not included in the 5.8-kb ori-β fragment that was shown here to function as an independent replicator at ectopic sites in uncloned pools of stably transfected DR12 cells (Fig. 3). However, given that the 3.2-kb sequence in the endogenous DHFR locus flanks the initiation zone at one end, one possibility is that it may promote replication over the entire zone by insulating it from position effects that would prevent its initiation activity. Although other possible functions for this element can also be imagined, this speculation makes specific predictions that could be tested experimentally.

In the endogenous DHFR locus, the three preferred replication start sites in the initiation zone have been suggested to represent redundant genetic elements (72). One prediction of this hypothesis is that each preferred site should be sufficient, on its own, to direct initiation of DNA replication. Our data show that this prediction is met by the ori-β region. Another prediction is that deletion of one of the preferred start sites from the endogenous locus might be compensated for by increased activity at the remaining two preferred start sites. Consistent with this prediction, deletion of a 4.5-kb ori-β sequence in the endogenous locus overlapping the 5.8-kb ori-β fragment used in our study failed to reduce initiation activity in a broad zone that retained ori-β′ and ori-γ sequences, as detected by two-dimensional gel electrophoresis (49). Since this 4.5-kb deletion encompassed two of the same regions in the 5.8-kb fragment that we found to be critical for initiation activity (pMCDΔAT and pAKO in Fig. 4), we speculate that the ori-β′ and ori-γ regions may be sufficient to direct initiation over the entire zone in the endogenous locus. It would be interesting to determine whether either the ori-β′ or ori-γ region could also serve as an independent chromosomal replicator in an ectopic location. The working model that the preferred start sites in the endogenous DHFR locus are redundant may provide additional insight into the complex patterns of replication intermediates observed for the 55-kb initiation zone by two-dimensional gel electrophoresis (26, 28, 41, 86, 89).

Is the ori-β region composed of essential modular elements? Studies of model systems have shown that replicators usually have a modular organization (reviewed in reference 22). The origin core consists of discrete elements: a DUE, an origin recognition element that contains binding sites for initiator proteins, and sometimes an AT-rich region. The core is often flanked by auxiliary elements that bind transcription factors and enhance the replication activity of the core element up to 1,000-fold.

In the ectopic DHFR ori-β region, at least two well-separated DNA sequence elements were critical for full activity (Fig. 4; Table 4). Deletion of the GA dinucleotide repeat in pMCDΔDNR or the central AT-rich sequence in pMCDΔAT reduced initiation activity nearly 10-fold. The markedly different sequence compositions of these two elements suggest that they probably serve different functions. Although these functions remain to be elucidated, several possible functions are suggested by previous studies. For example, GA dinucleotide repeats direct the establishment of a functionally important nucleosomal array in the transcription control region upstream of a *Drosophila* heat shock gene (61) and could play such a role in the ectopic DHFR ori-β region. The central AT-rich region that was deleted in pMCDΔAT harbors a potential DUE (16) and sequences homologous to a cell cycle-dependent protein footprint in the human lamin B2 (1, 25, 29, 39) and to the ORC binding sites in ACE3 (6, 80). Either DNA unwinding or protein binding could account for the requirement for the central AT-rich region in the 5.8-kb ori-β fragment. Alternatively, either or both of these two deletions could alter the spacing between flanking sequence elements, which may be critical for replication initiation.

A deletion of only 4 bp in the 5.8-kb ori-β fragment (pAKO) cut its initiation activity by half (Fig. 4D). Although this result was initially quite surprising, inspection of the DNA sequences around the deletion suggests at least two possible explanations for the striking effect of this mutation. The mutation removed 4 GC bp from a 6-bp run of GC sequence, one of only two such GC hexanucleotides in an AT-rich region of 1.2 kb between the pMCDΔAT and pMCDΔDNR mutations (Fig. 4A). Maintenance of this stretch of GC may be important for full initiation activity. Another possibility is that the 4-bp deletion altered the spacing between flanking modular elements that must cooperate to initiate replication. For example, insertions of approximately half of a DNA helical turn between neighboring elements in the SV40 early promoter was reported to decrease transcription activity by about 90%, whereas separation of the elements by a full helical turn was consistently less detrimental (81). Interestingly, the 4-bp deletion in pAKO resides just three nucleotides downstream of an AT-rich element that is homologous to ORC-binding sites in ACE3 (Fig. 4A) and may bind to hamster ORC protein (cited in reference 12). If ORC binding in this region indeed plays a role in ori-β activity, the 4-bp deletion may disrupt ORC interactions with flanking elements such as the stably bent region and the RIP60-binding sites whose functional importance remains unknown (15, 16).

In contrast with the other mutations, deletion of the upstream T-rich element (pMCDΔTR) more than tripled the activity of the DHFR ori-β IR (Fig. 4D), indicating that the T-rich element is not required for activity of the 5.8-kb fragment in ectopic sites. The deleted sequences encompassed an Alu repeat, a previously described cell cycle-dependent nuclease-hypersensitive site, and sequences homologous to ORC-binding sites in ACE3 and to *S. pombe* ARS elements (Fig. 4A), indicating that none of these elements is essential for ori-β activity. One possible interpretation of these data is that the T-rich element may limit the initiation activity of the ori-β region, either only in the ectopic fragment or also in the endogenous locus. Deletion of the T-rich element might eliminate a protein binding site that competes with the proposed ORC-binding site (12) immediately downstream from the deletion (Fig. 4A, checkered boxes) or possibly enhance cooperation between flanking DNA sequence elements that contribute to replicator activity. Alternatively, the deletion may affect chromatin structure, increasing protein access to the origin or promoting unwinding at the ori-β IR.

In summary, the results presented here strongly suggest that DNA sequences in the 5.8-kb DHFR fragment are sufficient to direct efficient initiation at the ori-β IR in multiple ectopic chromosomal sites and that initiation activity depends on discrete genetic elements located at or near the IR. Further characterization of these elements will be required to confirm their proposed modular nature and to elucidate their biochemical functions.

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